

# Radioimmunoassay for quantifying antibody to *N. gonorrhoeae* in human sera

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Internationally, the incidence of gonococcal disease, both genital and non-genital, has been increasing. For example, the rate in Denmark has risen from approximately 100 cases per 100,000 population in 1965 to a peak of over 300 cases per 100,000 in 1971 (Lind, 1973). The annual incidence of reported cases of gonococcal disease in the United States has risen in the last 8 years to over 400 cases per 100,000 population (USPHS, 1973). Furthermore, the various indices for enumerating cases of gonococcal disease have probably given grossly low rates due to under-reporting, inaccurate diagnosis, and failure to detect infected but asymptomatic individuals.

Detection of the asymptomatic carrier remains perhaps the most important element in controlling outbreaks of gonococcal disease. In addition to spreading primary gonorrhoea, the asymptomatic carrier population comprises the reservoir from which the majority of disseminated infections arise. The frequency of asymptomatic genital gonorrhoea in the female has been well documented (Lucas, Price, Thayer, and Schroeter, 1967), but asymptomatic gonococcal disease in males, once thought to be non-existent, has been detected with an increased frequency in recent years (Enfors, Eriksson, Kaaman, and von Krogh, 1973). Recently, *Neisseria gonorrhoeae* was isolated from 2.2 per cent. of 2,628 sexually active servicemen, and 68 per cent. of the culturally positive men were asymptomatic (Handsfeld, Lipman, Harnisch, Tronca, and Holmes, 1974).

Non-genital gonococcal infections, both asymptomatic and symptomatic, are difficult to diagnose accurately. In one series, patients originally diagnosed as cases of ulcerative proctitis had, in fact, gonococcal proctitis (Kilpatrick, 1972). In addition, gonococcal infection of the pharynx, once thought to be very rare, has been diagnosed with increasing frequency (Ratnatunga, 1972). Results from another investigation of 110 patients in whom pharyngeal cultures were positive for gonococci, showed that 87 (79 per

cent.) were without symptoms, whereas seventeen (15 per cent.) had minor sore throats, and only six (5 per cent.) had acute febrile tonsillitis (Bro-Jørgensen and Jensen, 1973). Similar results have been obtained by other investigators (Stolz and Schuller, 1974).

This brief review of the incidence of asymptomatic and atypical gonococcal disease is intended to point out the need to develop a diagnostic method which would be an alternative or a supplement to culturing gonococci from every potentially infected patient. It was felt that an inexpensive serodiagnostic procedure would be the most practical test for this purpose.

This paper describes the development of a radioimmunoassay (RIA) procedure which could detect antibodies specific for *N. gonorrhoeae* in the serum of infected patients. The efficiency of the procedure was evaluated preliminarily from the data collected by assay of the reactivity of 444 human sera with <sup>14</sup>C-labelled gonococcal antigen. The results showed that this technique, or a modification thereof, could be employed to screen sera from human cohorts which have a high risk of contracting gonorrhoea; it would aid in the detection of asymptomatic carriers and in diagnosing cases of non-genital gonococcal disease. A preliminary report on this work has already been presented (Luoma and Rudbach, 1975).

## Material and methods

### GONOCOCCAL STRAIN

*N. gonorrhoeae* strain F62 was obtained from Dr. D. S. Kellogg, Jr., Center for Disease Control, Atlanta, Georgia. The organisms were stored in lyophilized form. During periods of frequent use, the cultures were maintained on GC Medium Agar Base (Difco) supplemented with 1 per cent. IsoVitalex (BBL). Type 1 colonies (Kellogg Peacock, Deacon, Brown, and Pirkle, 1963) were selected and transferred daily.

### PREPARATION OF <sup>14</sup>C-LABELLED ANTIGEN EXTRACT

Biosynthetically labelled gonococcal antigen was prepared from organisms grown with D-glucose-UL-<sup>14</sup>C (ICN).

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One mCi of  $^{14}\text{C}$ -glucose was added per litre of GC medium base broth supplemented with 5 per cent. calf serum. The cultures were incubated for 48 hrs at 38°C. with mild shaking on a wrist-action shaker. Each flask was gassed with a mixture of 83 per cent. nitrogen, 10 per cent. carbon dioxide, and 7 per cent. oxygen. Cells were harvested by centrifugation and washed with 0.1 M. Tris-HCl buffer, pH 8.0 (Tris). The washed cells from one litre of medium were suspended in 25 ml. of 2 per cent. sodium deoxycholate in Tris and allowed to stand at room temperature for 1.5 hrs. The disrupted cells were then centrifuged for 30 min. at  $27,000 \times G$  and 25°C. Strands of nucleic acid were removed with a glass rod and the antigen was precipitated by decanting the supernatant fluid into six volumes of 95 per cent. ethanol and allowing the precipitate to develop overnight at room temperature. Additional nucleic acid floccules were removed from the precipitate by filtration on to gauze. The filtrate was centrifuged at  $1500 \times G$  for 30 min. to separate out an insoluble fraction. This pellet was suspended in 15 ml. distilled water and recentrifuged at  $27,000 G$  for 30 min. to sediment a particulate fraction. The supernatant fluid from the final centrifugation (containing the soluble antigen) was transferred to dialysis tubing and dialysed for 5 days against daily changes of distilled water at 4°C. After dialysis, the antigen was lyophilized.

#### SOURCES OF HUMAN SERA

Sera\* were obtained from patients who were culturally positive for *N. gonorrhoeae* (acute phase sera) and from patients who had been treated previously for gonorrhoea and were now culturally negative (convalescent sera). Negative control sera were obtained from several sources. Sera from both sexes were collected at a county health department out-patient facility from individuals who denied during premarital interviews any history of gonococcal infection. These individuals were not cultured and no other information was available on them. Well documented samples of negative control sera were obtained from male volunteers at a local military installation. The blood samples were drawn during routine physical examination. A second batch of well documented negative sera was obtained by a local gynaecologist. Serum samples included in this group were from women who gave no past history of gonorrhoea and were culturally negative for *N. gonorrhoeae*. All samples were stored at -20°C.

In addition to the individual serum samples described above, two serum pools were prepared and standardized. Both these pools were made by mixing, in varying proportions, sera obtained from a local blood bank. One pool was prepared so that when it was reacted with the gonococcal antigen in the radioimmunoassay it gave an activity ratio similar to that obtained with serum samples from anatomically vaginal females; this was designated V-1. The second serum pool, designated V-2, was

prepared in a similar manner so that it assayed with a ratio which approximated the mean value obtained from a series of sera from well documented, culturally negative male volunteers.

#### PREPARATION OF ANTIGLOBULIN

Antiserum against human gamma globulin was obtained from New Zealand white rabbits which had been injected repeatedly with the Cohn Fraction II (gamma globulin fraction) of human plasma (Cutter Laboratories) emulsified in complete Freund adjuvant (Difco). Serum samples from separate bleedings were pooled, heat inactivated, and stored at -20°C. A quantitative precipitation assay was performed to determine the equivalence point of each antiserum pool with human plasma.

#### RADIOIMMUNOASSAY

The reaction mixture for assaying antigenococcal activity in the serum samples consisted of 500  $\mu\text{l}$ . phosphate buffered saline (0.15 M NaCl; 0.0033 M  $\text{PO}_4$ ; pH 7.2) (PBS), 10 or 20  $\mu\text{l}$ . undiluted serum, and 10  $\mu\text{l}$ . labelled gonococcal antigen solution containing 1  $\mu\text{g}$ . antigen in a 12 ml. conical centrifuge tube. The serum and antigen solutions were dispensed with microlitre pipettes. The reaction mixture was incubated for 30 min. at 37°C. After the initial incubation, 0.25 ml. rabbit antihuman gamma globulin was added, and the mixture was re-incubated for 30 min. at 37°C. and overnight at 4°C. The precipitate containing the bound antigen was recovered by centrifugation at 1,000 G. for 30 min. at 4°C. The supernatant fluid was decanted and saved for analysis. The precipitate was washed twice with ice-cold PBS and drained by inverting the tubes for 15 min. The precipitate was digested by the addition of 1 ml. of sample solubilizer (Soluene, Packard Instrument Co.) followed by incubation for 4 hrs at 56°C. and overnight at room temperature. 100  $\mu\text{l}$ . of each supernatant fluid were transferred to a scintillation vial containing 1 ml. Soluene and, the vials were incubated as before. After solubilization of the protein in the supernatant fluids, 10 ml. scintillation fluid were added and each vial was mixed gently. The solubilized precipitates were transferred quantitatively to counting vials with 10 ml. scintillation fluid. Each sample was counted in a Nuclear Chicago Unilux III scintillation counter at optimal voltage and window settings for  $^{14}\text{C}$ . The composition of this scintillation cocktail has been described by Rudbach and Weber (1971).

The activity ratio (bound to unbound antigen) for each serum was determined by the following formula:

$$\text{Activity ratio} = \frac{(\text{cpm in precipitate} - \text{background cpm})}{(\text{cpm in } 100\mu\text{l. supernatant fluid} - \text{background cpm}) + (\text{cpm in precipitate} - \text{background cpm})}$$

#### Results

To establish the levels of statistical confidence which could be ascribed to values obtained from the radio-immunoprecipitation assay for gonorrhoea, the following experiment was performed. Five samples of serum were each assayed ten times for reactivity with the gonococcal antigen, and the resulting activity ratios were analysed statistically (Table I).

\*Serum specimens were obtained through the generous help of the following individuals: Linda Rives and Jan Iverson, Missoula County Health Department, Missoula, Montana; Dr. F. D. Anderson, Professional Village, Missoula, Montana; John Hall and Gene Johnson, Montana Air National Guard, Great Falls, Montana; and Dr. J. D. Dyckman, Department of Public Health, Houston, Texas.

TABLE 1 *Statistical analysis of activity ratios given by selected sera during repetitive radioimmunoprecipitation assays with gonococcal antigen*

Serum Number	Mean	Range	Standard error	
			Value	Percentage of the mean
1	0.265	0.303-0.223	0.0081	3.1
63	0.232	0.361-0.136	0.0194	8.4
86	0.312	0.377-0.248	0.0126	4.0
109	0.231	0.336-0.187	0.0158	6.8
286	0.261	0.389-0.213	0.0174	6.7
			Mean	5.8

The standard errors varied from 3.1 to 8.4 per cent. of their respective means, with a mean variation of 5.8 per cent. of the mean. In all subsequent assays, any activity ratios which fell within two standard errors of the lines of division between positive and negative were considered indecisive.

Sera from 444 individuals were tested in the radioimmunoprecipitation assay. The subjects' sera were divided into groups and the activity ratios were compared in a scatter diagram format (Figure). Data obtained with sera from males and females were listed separately because of differences in their respective baseline reactivities in the assay. Normal sera from female subjects were usually less reactive in this assay than sera from males. Therefore, the division between positive and negative reacting sera was set at an activity ratio of 0.200 for females and 0.250 for males. Two serum pools, V-1 for females and V-2 for males, were prepared as described under 'Material and methods'. These two sera were included each time an assay was run and their activity ratios were taken as the division points between positive and negative; also, the activity ratios of V-1 and V-2 were used to normalize activity ratios obtained with batches of sera tested in assays with different preparations of antigen. The activity ratios of V-1 and V-2 sera were adjusted to 0.200 and 0.250 respectively, and the activity ratios of the serum samples from female and male subjects assayed at the same time were also adjusted proportionately.

The Figure shows that the majority of sera from individuals who were infected with *N. gonorrhoeae* for longer than 7 days or who were convalescent for less than 6 months gave activity ratios in the radioimmunoprecipitation assay greater than the demarcation lines between positive and negative (Table II). Of 166 serum samples from culturally positive males, 154 (93 per cent.) were diagnosed correctly. Eight of the 166 sera fell within two standard errors of the demarcation line and diagnoses based on their activity ratios were indecisive. Only four of the sera from culturally positive males displayed a clear-cut negative activity in the RIA.

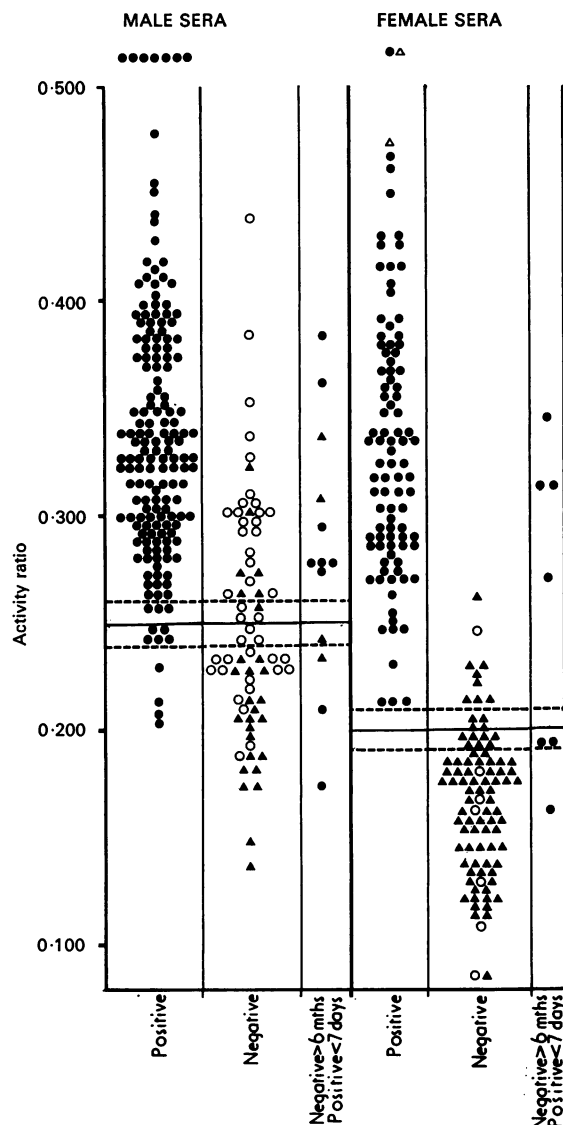


FIGURE *Comparison of activity ratios in RIA of sera from patients with varying histories of gonococcal disease*

- culturally positive for gonorrhoea, with symptoms
- △—culturally positive, asymptomatic carrier
- ▲—well-defined negative (Group 2)
- ill-defined negative (Group 1)

Negative control sera from males were obtained from two sources: an out-patient county health facility (Group 1) and a local military installation (Group 2). Group 1 sera were collected during premarital interviews and were designated as negative by the subjects' responses to a single question; these individuals were not cultured. Out of 42 of these ill-defined Group 1 control sera, 21 gave false

TABLE II *Summary interpretation of reactivity of sera with gonococcal antigen in a radioimmunoprecipitation assay*

Sex of patient	Patient history	Number tested	RIA diagnosis		Percentage correct
			Diagnosis	Number	
Male	Positive <sup>a</sup>	166	positive <sup>b</sup> negative <sup>c</sup> indecisive <sup>d</sup>	154 4 8	93
	Negative <sup>e</sup> Group 1 <sup>f</sup>	42	positive <sup>b</sup> negative <sup>c</sup> indecisive <sup>d</sup>	21 15 6	36
	Negative <sup>e</sup> Group 2 <sup>g</sup>	28	positive <sup>b</sup> negative <sup>c</sup> indecisive <sup>d</sup>	6 21 1	75
	Total of positive and negative	236	correct incorrect indecisive	190 31 15	81
	Total of positive and Group 2	194	correct incorrect indecisive	175 10 9	90
Female	Positive <sup>a</sup>	95	positive <sup>h</sup> negative <sup>i</sup> indecisive <sup>j</sup>	95 0 0	100
	Negative <sup>k</sup>	93	positive <sup>h</sup> negative <sup>i</sup> indecisive <sup>j</sup>	9 73 11	78
	Total of positive and negative	188	correct incorrect indecisive	168 9 11	89
Total	Positive	261	positive negative indecisive	249 4 8	95
	Negative with Group 1	163	positive negative indecisive	36 109 18	67
	Negative without Group 1	121	positive negative indecisive	15 94 17	78
	Total of positive and negative	424	correct incorrect indecisive	358 40 26	84
	Total of positive and Group 2	382	correct incorrect indecisive	343 19 20	90

<sup>a</sup>culturally positive;<sup>b</sup>activity ratio greater than 0.262;<sup>c</sup>activity ratio less than 0.238;<sup>d</sup>activity ratio between 0.238 and 0.262;<sup>e</sup>negative on interview, no culture done;<sup>f</sup>ill-defined negative;<sup>g</sup>well-defined negative;<sup>h</sup>activity ratio greater than 0.212;<sup>i</sup>activity ratio less than 0.188;<sup>j</sup>activity ratio between 0.188 and 0.212;<sup>k</sup>virginal or negative on culture

positive reactivity. However, negative control sera obtained from the military installation, which had detailed medical histories on each man (Group 2 control sera) demonstrated a false positive rate of only 21 per cent. In all, 75 per cent. of the Group 2

control sera were designated correctly as negative. In total, 81 per cent. of the serum samples from males were given a designation by the RIA results which correlated with the subjects' histories of gonorrhoea; this value was increased to 90 per cent. when the ill-defined negative sera were eliminated from calculations.

A 100 per cent. detection rate was obtained when sera from actively infected females were assayed. Of major interest were the two sera from females which exhibited no symptoms (Figure). These sera were among those which gave the highest activity ratios of all sera from female subjects. Although it was a small sample size, the activity ratios shown by these two sera might be indicative of titres which could be expected in asymptomatic female carriers. Sera from females with no history of gonorrhoea were designated correctly as negative by RIA in 78 per cent. of cases. In total, a correct correlation for both positive and negative cases was made with over 89 per cent. of serum samples from females (Table II). An incorrect diagnosis for females was extrapolated from RIA results in less than 5 per cent. of cases.

A problem was encountered in correlating activity ratios of sera with histories in a particular group of subjects. These individuals, both male and female, were convalescent from gonorrhoea for more than 6 months or had been primarily infected with gonorrhoea for less than 7 days. (Figure). Apparently, the individual variation in rate of production and deterioration of circulating antibody specific for the gonococcus at these temporal extremes of the immune response was the source of the problem. If these twenty sera were omitted from further consideration, 84 per cent. of the remaining 424 samples (from both males and females) could be correlated correctly by RIA analysis with the subjects' histories. Again, the overall correct diagnosis was increased to 90 per cent. when the ill-defined negative sera were excluded from the results.

## Discussion

Radioimmunoprecipitation has been observed to be an extremely sensitive and specific method for detection of antibody (*British Medical Bulletin*, 1973). The RIA procedure described and evaluated in this paper seems to hold promise as a means to screen for gonococcal disease. In general, it was found that sera from acutely infected (longer than 1 week) and convalescent (shorter than 6 months) patients reacted more strongly with the gonococcal antigen in the RIA than did control sera. This reactivity correlated with previous work which demonstrated the temporal antibody response to the gonococcus (Cohen, Kellogg, and Norins, 1969). Antibodies to *N. gonorrhoeae* appeared at 5 to 7 days with the peak titres occurring at 14 days. The titres dropped

rapidly and seemed to have dissipated by 6 months (Cohen and others, 1969).

During the course of these assays an important observation was made. Normal male sera routinely demonstrated higher reactivity with the gonococcal antigen than did the female sera. No explanation has been found for this phenomenon. However, recognition of this differential reactivity of sera from the two sexes facilitated the interpretation of raw data from the RIA. If this higher reactivity of male sera with gonococcal antigens occurred in other types of assays, it probably resulted in some misinterpretations of results in previous studies.

The rate of false positive reactions in the male control sera of Group 1 (Table II) was of considerable concern. These control sera were obtained from outpatient public health facilities and were assigned negative status by the word of the patient; that is to say, the patient was never cultured for *N. gonorrhoeae*. However, in the circumstances of the interview (premarital) and according to the technicians who took the blood samples, there was reason to doubt the truth of the men's statements. We felt that this high rate of false positive reactions was not indicative of the actual false positive rate. Therefore, a better defined source of male control sera was sought. Group 2 sera were obtained from a local military installation which had the medical histories of each of the patients. Sera from this group demonstrated a much lower false positive rate (21 per cent.), which was close to that obtained with the female control sera. The female sera, for the most part, were collected by a local gynaecologist who routinely cultured all of his patients and maintained an excellent history on them; this confirmed the negative status of the individuals. The false positive rate obtained with these sera compared favourably to the rates obtained by other serodiagnostic methods (*British Medical Journal*, 1972). We are confident that our negative control sera reflected the true normal reactivity of the RIA for gonorrhoea. This is because all negative control sera used were obtained from adults rather than from adolescents. This avoided the valid criticism that children might lack exposure to micro-organisms which could induce antibodies cross-reacting with the gonococcal antigen.

The data presented in this paper have demonstrated efficiency rates for serodiagnosis of gonorrhoea which were comparable to or more satisfactory than the results reported previously for a radioimmunoassay in which purified pili were employed as the antigen

(Buchanan, Swanson, Holmes, Kraus, and Gotschlich, 1973). Attempts are being made in our laboratory to purify the antigen contained in the supernatant extract. Also, the assay is being modified so that more easily quantifiable radioisotopes can be used and simplified methods can be employed for distinguishing immunoglobulin-bound antigen from unbound antigen. It is hoped that a modification of the procedure described above will be useful in the routine serodetection of gonococcal carriers.

### Summary

A radioimmunoassay has been developed for the serodiagnosis of gonorrhoea. When 382 human sera from acutely infected and well documented control subjects were assayed by this procedure, 90 per cent. were diagnosed correctly, 5 per cent. were diagnosed incorrectly, and in 5 per cent. the results were indecisive.

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